Supplementary Appendix

This appendix has been provided by the authors to give readers additional information about their work.

Supplement to: Drilon A, Laetsch TW, Kummar S, et al. Efficacy of larotrectinib in *TRK* fusion–positive cancers in adults and children. N Engl J Med 2018;378:731-9. DOI: 10.1056/NEJMoa1714448

SUPPLEMENTARY APPENDIX

Supplement to: Drilon A, Laetsch TW, Kummar S, et al. Efficacy of Larotrectinib in TRK Fusion-Positive Adult & Pediatric Cancers

Contents **Page number**

TRK Fusion Screening Assays and Methodologies

TRK fusions were identified by next-generation sequencing or by fluorescence in situ hybridization as routinely obtained by each participating site. All testing was performed in a Clinical Laboratory Improvement Amendments (CLIA)-certified laboratory or equivalent for laboratories located outside of the United States. The most common local assays used included those performed by Foundation Medicine (Cambridge, MA, USA),[1,2] Memorial Sloan Kettering Cancer Center (New York, NY, USA),[3,4] and University of Washington (Seattle, WA, USA)[5] using previously published methods. In total, these labs accounted for 78% (43/55) of patients accrued with the remaining laboratories contributing two or fewer patients each.

Sequencing and IHC Methods (For Primary/Acquired Resistance Testing)

Pan-TRK Immunohistochemistry

Formalin-fixed paraffin embedded tumor tissue was stained with a pan-TRK rabbit monoclonal antibody (clone EPR17341, Abcam, Cambridge, MA) that recognizes the C-terminal region of TRKA, TRKB, and TRKC. Staining was performed on the Leica Bond III autostainer platform (Leica Biosystems Inc., Buffalo Grove, IL) at Paradigm Diagnostics (Phoenix, AZ). Heat-induced epitope retrieval was performed with the BOND Epitope Retrieval Solution 2 (Leica Biosystems Inc., Buffalo Grove, IL) followed by incubation with 4 µg/ml of the pan-TRK antibody. Primary antibody binding was visualized using the Leica Bond Polymer Refine Detection (DAB) kit (Leica Biosystems Inc., Buffalo Grove, IL).

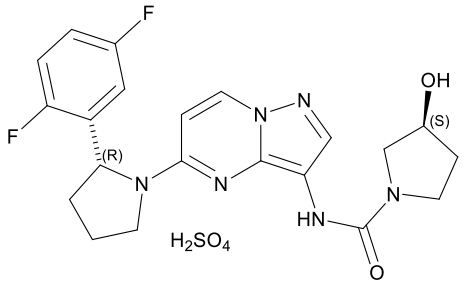
Cell-Free DNA Analysis

Analysis of cell-free (cf)DNA was performed at Guardant Health, Inc. (Redwood City, CA) using the G360 panel[6] on plasma isolated from patient blood samples. The G360 panel detects single nucleotide variants in 73 genes (including select regions of *NTRK1* and *NTRK3*) and fusions in 6 genes (including some fusions involving *NTRK1*), as well as additional types of variants. Following cfDNA isolation, DNA was barcoded for library preparation. Libraries were amplified and enriched for the target genes using biotinylated custom baits, which in turn were pair-end sequenced on an Illumina HiSeq 2500 (Illumina, Inc., San Diego, CA). Variants were quantified from the resulting sequence data using Guardant's proprietary bioinformatics algorithms.

Tissue Analysis

Analysis of tumor samples was performed at Foundation Medicine, Inc. (Cambridge, MA) using the Foundation One panel[1] or by Memorial Sloan Kettering-integrated mutation profiling of actionable cancer targets (MSK-IMPACT).[3]

Table S1. Larotrectinib Characteristics



Characteristic		Value
Enzyme Affinity (IC_{50})	TRKA/B/C	5.3-11.5 nM
Enzyme Off Rate (half-life)[1]	TRKA	45 min
	pTRKA	160 min
Cellular Potency (IC_{50}), Engineered constructs	TRKA/B/C	9.8-25 nM
Cellular Potency (IC_{50}), *TPM3-NTRK1*	KM12 line	3.5 nM
Cellular Potency (IC_{50}), *MPRIP-NTRK1*	Cuto3.29 line	59 nM
Cellular Potency (IC_{50}), *TRIM24-NTRK2*	BAF cells	35 nM
Cellular Potency (IC_{50}), *ETV6-NTRK3*	Mo91 line	1 nM
Selectivity (229 kinases tested)		≥100-fold
Selectivity (80 non-kinase targets)		≥1000-fold
Solubility (mg/mL)	pH <2	>15
	pH 3	2.0
	pH 5	1.0
	pH 6.8	1.0
	pH 7.4	1.2
Oral Bioavailability	Mouse	53%
	Rat	33%
	Dog	66%
	Monkey	100%
Plasma Protein Binding (Unbound fraction)	Mouse	40%
	Rat	37%
	Dog	39%
	Monkey	33%
	Human	30%
TRK systemic target coverage at 100mg BID Cmax	Human	98%
TRK CNS target coverage at 100mg BID Cmax	Human	95%
Brain penetration (Ommaya CSF/unbound plasma ratio)	Human	28%
Brain penetration (unbound brain/ unbound plasma ratio by microdialysis)	Rat	4%
CYP450 Inhibition (IC_{50})	CYP1A2	>300 μM
	CYP2B6	>300 μM

Characteristic		Value
	CYP2C8	180 µM
	CYP2C9	>300 µM
	CYP2D6	>300 µM
	CYP3A4	>300 µM (weak TDI[2])

[1]Larotrectinib was tested in kinetic studies using a Biacore S51 surface plasmon resonance machine (Biosensor Tools, Salt Lake City, UT).

[2]TDI=time-dependent inhibition.

Table S2. Patient Demographics by Study

Characteristic	Study			
	Adults Phase 1 (n=8)	**Pediatric Phase 1 (n=12)**	**Adolescent/ Adult Phase 2 (n=35)**	**All patients (n=55)**
Sex — no. (%)				
Male	7 (88)	6 (50)	16 (46)	29 (53)
Female	1 (13)	6 (50)	19 (54)	26 (47)
Race — no. (%)				
White	8 (100)	5 (42)	24 (69)	37 (67)
Asian	0	0	2 (6)	2 (4)
Black or African American	0	0	2 (6)	2 (4)
All others	0	7 (58)	7 (20)	14 (25)
Median age (range), years	48.0 (28.0–66.0)	1.8 (0.3–12.0)	59.0 (24.0–76.0)	45.0 (0.3–76.0)
Age group — no. (%)				
<2 years	0	6 (50)	0	6 (11)
2–5 years	0	5 (42)	0	5 (9)
6–14 years	0	1 (8)	0	1 (2)
15–39 years	3 (38)	0	9 (26)	12 (22)

≥40 years	5 (63)	0	26 (74)	31 (56)
ECOG performance status — no. (%)				
0	4 (50)	9 (75)	11 (31)	24 (44)
1	4 (50)	2 (17)	21 (60)	27 (49)
2	0	1 (8)	3 (9)	4 (7)
Number of prior systemic chemotherapies — no. (%)				
0–1	3 (38)	7 (58)	17 (49)	27 (49)
2	1 (13)	2 (17)	6 (17)	9 (16)
≥3	4 (50)	3 (25)	12 (34)	19 (35)
Tumor type — no. (%)				
Salivary gland	3 (38)	0	9 (26)	12 (22)
Soft tissue sarcoma, other[†]	1 (13)	5 (42)	5 (14)	11 (20)
Infantile fibrosarcoma	0	7 (58)	0	7 (13)
Thyroid	1 (13)	0	4 (11)	5 (9)
Colon	0	0	4 (11)	4 (7)
Lung	1 (13)	0	3 (9)	4 (7)
Melanoma	0	0	4 (11)	4 (7)
GIST	2 (25)	0	1 (3)	3 (5)
Cholangiocarcinoma	0	0	2 (6)	2 (4)

Appendix	0	0	1 (3)	1 (2)
Breast	0	0	1 (3)	1 (2)
Pancreas	0	0	1 (3)	1 (2)
Disease status at study enrollment — no. (%)				
Metastatic	8 (100)	4 (33)	33 (94)	45 (82)
Locally advanced	0	8 (67)	2 (6)	10 (18)
CNS metastases — no. (%)				
No	8 (100)	12 (100)	34 (97)	54 (98)
Yes	0	0	1 (3)	1 (2)
TRK gene fusion — no. (%)				
NTRK1	2 (25)	6 (50)	17 (49)	25 (45)
NTRK2	0	1 (8)	0	1 (2)
NTRK3	6 (75)	5 (42)	18 (51)	29 (53)
TRK fusion gene — no. (%)				
ETV6-NTRK3	6 (75)	5 (42)	17 (49)	28 (51)
TPM3-NTRK1	0	4 (33)	5 (14)	9 (16)
LMNA-NTRK1	1 (13)	0	4 (11)	5 (9)
IRF2BP2-NTRK1	0	0	2 (6)	2 (4)
SQSTM1-NTRK1	0	1 (8)	1 (3)	2 (4)

CTRC-NTRK1	0	0	1 (3)	1 (2)
GON4L-NTRK1	0	0	1 (3)	1 (2)
PDE4DIP-NTRK1	0	1 (8)	0	1 (2)
PLEKHA6-NTRK1	0	0	1 (3)	1 (2)
PPL-NTRK1	0	0	1 (3)	1 (2)
STRN-NTRK2	0	1 (8)	0	1 (2)
TPM4-NTRK3	0	0	1 (3)	1 (2)
TPR-NTRK1	1 (13)	0	0	1 (2)
TRIM63-NTRK1	0	0	1 (3)	1 (2)

CNS denotes central nervous system, ECOG Eastern Cooperative Oncology Group, GIST gastrointestinal stromal tumor.

[†]Morphologic appearances of other soft tissue tumors: myopericytoma (n=2); not otherwise specified (n=2); peripheral nerve sheath (n=2); spindle cell (n=3); infantile myofibromatosis (n=1); inflammatory myofibroblastic tumor of kidney (n=1).

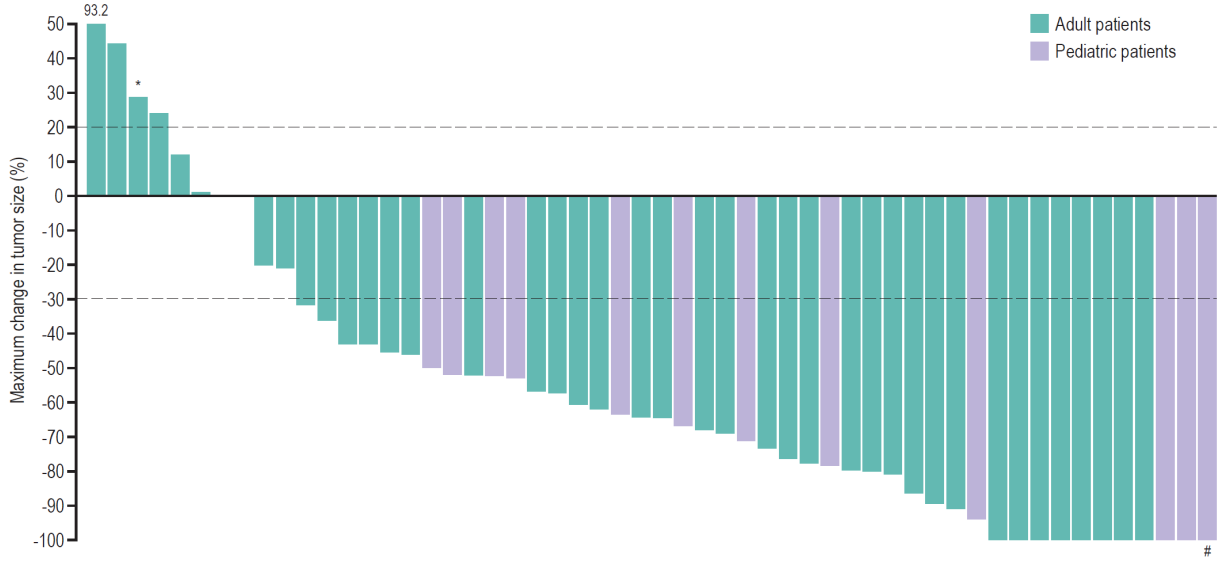
Table S3. Resistance Findings

Clinical Trials	Primary Diagnosis	TRK Fusion	*NTRK* Kinase Domain Mutation(s)
Adult phase 1	Lung	*TPR-NTRK1*	NTRK1 G595R and NTRK1 G667S
Adolescent/adult phase 2	Colon	*LMNA-NTRK1*	NTRK1 G595R
Adolescent/adult phase 2	Cholangiocarcinoma	*LMNA-NTRK1*	NTRK1 F589L
Adolescent/adult phase 2	Pancreas	*CTRC-NTRK1*	NTRK1 A608D
Adolescent/adult phase 2	Thyroid	*IRF2BP2-NTRK1*	NTRK1 G595R
Adolescent/adult phase 2	Colon	*TPM3-NTRK1*	NTRK1 G595R and NTRK1 F589L
Adolescent/adult phase 2	Salivary gland	*ETV6-NTRK3*	Not tested
Adolescent/adult phase 2	Soft tissue sarcoma	*TPM3-NTRK1*	NTRK1 G595R
Adolescent/adult phase 2	GIST	*ETV6-NTRK3*	NTRK3 G623R and NTRK3 G696A
Pediatric phase 1	IFS	*ETV6-NTRK3*	NTRK3 G623R

*IFS denotes infantile fibrosarcoma and GIST gastrointestinal stromal tumor.

Figure S1. Waterfall Plots

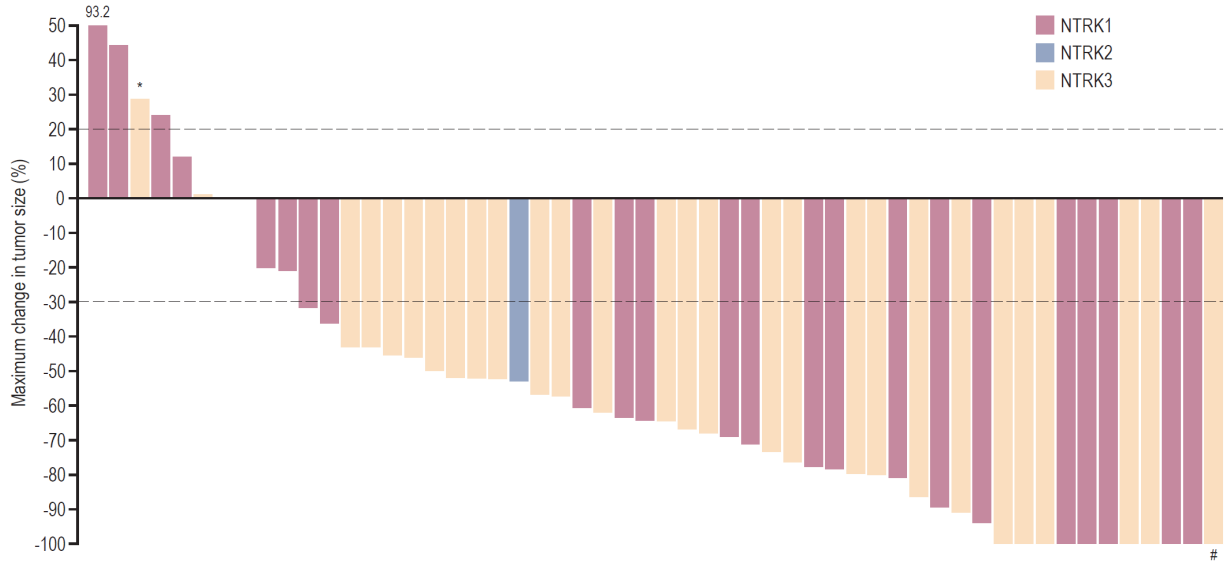
(a) Age



*Patient had TRK solvent front resistance mutation (NTRK3 G623R) at baseline due to prior therapy. #Pathologic CR.

NOTE: One patient not shown here. Patient experienced clinical deterioration and no post-baseline tumor measurements were recorded.

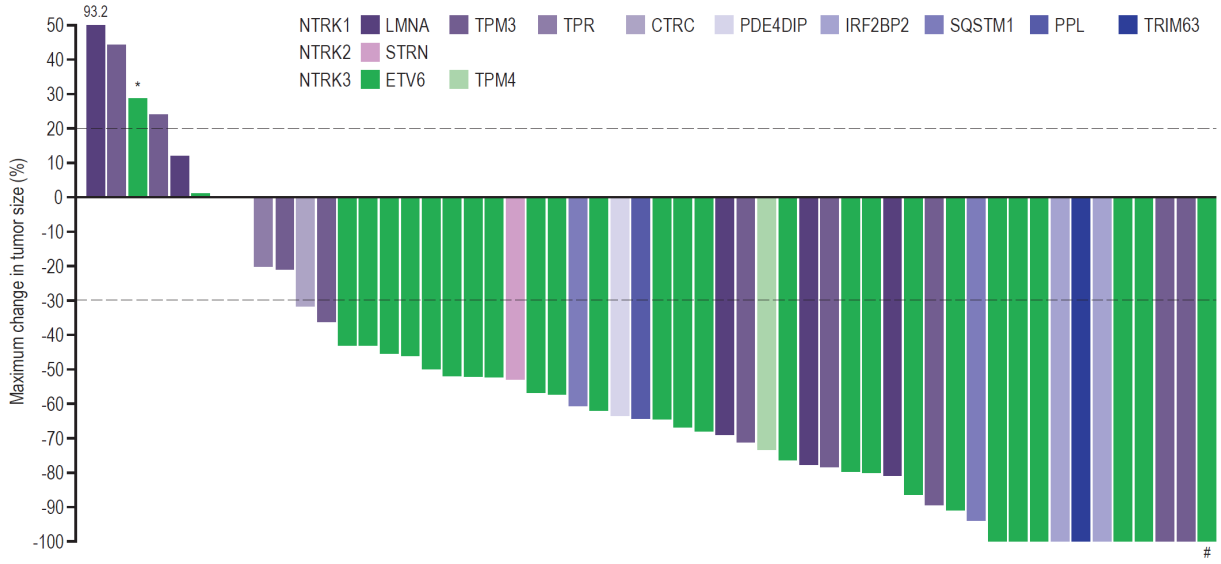
(b) *NTRK* Gene



*Patient had TRK solvent front resistance mutation (NTRK3 G623R) at baseline due to prior therapy. #Pathologic CR.

NOTE: One patient not shown here. Patient experienced clinical deterioration and no post-baseline tumor measurements were recorded.

(c) TRK Fusion Partner



*Patient had TRK solvent front resistance mutation (NTRK3 G623R) at baseline due to prior therapy. #Pathologic CR.

NOTE: One patient not shown here. Patient experienced clinical deterioration and no post-baseline tumor measurements were recorded.

Supplementary References

1. Frampton GM, Fichtenholtz A, Otto GA, et al. Development and validation of a clinical cancer genomic profiling test based on massively parallel DNA sequencing. Nat Biotechnol 2013;31:1023-31.
2. He J, Abdel-Wahab O, Nahas MK, et al. Integrated genomic DNA/RNA profiling of hematologic malignancies in the clinical setting. Blood 2016;127(24):3004-14.
3. Cheng DT, Mitchell TN, Zehir A, et al. Memorial Sloan Kettering-Integrated Mutation Profiling of Actionable Cancer Targets (MSK-IMPACT): A Hybridization Capture-Based Next-Generation Sequencing Clinical Assay for Solid Tumor Molecular Oncology. J Mol Diagn 2015;17:251-64.
4. Zheng Z, Liebers M, Zhelyazkova B, et al. Anchored multiplex PCR for targeted next-generation sequencing. Nat Med 2014;20:1479-84.
5. Pritchard CC, Salipante SJ, Koehler K, et al. Validation and implementation of targeted capture and sequencing for the detection of actionable mutation, copy number variation, and gene rearrangement in clinical cancer specimens. J Mol Diagn 2014;16:56-67.
6. Lanman RB, Mortimer SA, Zill OA, et al. Analytical and clinical validation of a digital sequencing panel for quantitative, highly accurate evaluation of cell-free circulating tumor DNA. PLoS One 2015;10:e0140712.